Assembly and localization of Toll-like receptor signalling complexes

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Abstract | Signal transduction by the Toll-like receptors (TLRs) is central to host defence against many pathogenic microorganisms and also underlies a large burden of human disease. Thus, the mechanisms and regulation of signalling by TLRs are of considerable interest. In this Review, we discuss the molecular basis for the recognition of pathogen-associated molecular patterns, the nature of the protein complexes that mediate signalling, and the way in which signals are regulated and integrated at the level of allosteric assembly, post-translational modification and subcellular trafficking of the components of the signalling complexes. These fundamental molecular mechanisms determine whether the signalling output leads to a protective immune response or to serious pathologies such as sepsis. A detailed understanding of these processes at the molecular level provides a rational framework for the development of new drugs that can specifically target pathological rather than protective signalling in inflammatory and autoimmune disease.

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The human genome encodes ten Toll-like receptors (TLRs) that recognize and respond to conserved microbial stimuli, known as pathogen-associated molecular patterns (PAMPs), and are therefore described as pattern recognition receptors (PRRs)[1]. By contrast, the *Drosophila melanogaster* protein Toll is activated by an endogenous ligand and has a role in fruit fly development, as well as in innate immunity[2]. The TLRs and Toll are type I transmembrane receptors with extracellular ligand-binding domains, a single membrane-spanning segment and a cytosolic Toll/IL-1R (TIR) domain. TLRs can be broadly subdivided into those that are localized at the cell surface and are activated by lipid and protein ligands, and those that signal in response to non-self nucleic acids from endosomal compartments (FIG. 1). Activating stimuli bind to the receptor ectodomain and induce the dimerization of the TIR domains that then act as a scaffold for downstream signal transducers. These adaptor proteins also have TIR domains and associate specifically with the receptor dimers through TIR–TIR interactions. The engagement of the adaptor proteins then promotes the assembly of higher-order complexes — namely, the helical 'Myddosome' complex for myeloid differentiation primary response protein 88 (MYD88)-dependent activation of nuclear factor-κB (NF-κB) and the 'Triffosome' for the activation of interferon (IFN)-regulatory factors (IRFs) by TIR domain-containing adaptor protein inducing IFNβ (TRIF). Although this general scheme for the activation of TLRs is well understood, the molecular mechanisms that are involved have only begun to be elucidated in the past five years. In this Review, we provide an overview of the TLR signalling pathway from a molecular perspective and highlight potential targets for the development of novel and specific anti-inflammatory therapies.

Ligand recognition and signal initiation

The ectodomains of TLRs comprise tandem leucine-rich repeats (LRRs), which are short motifs that fold into a characteristic solenoid structure (FIG. 2). Structural analysis has uncovered the molecular basis of ligand recognition by Toll and TLRs. These studies have revealed three distinct activation mechanisms for *D. melanogaster* Toll, cell-surface TLRs and endosomal TLRs that produce topologically similar activated complexes.

PAMP-induced dimerization. As illustrated in FIG. 1, the cell-surface TLRs are monomeric but form active homodimers or heterodimers when exposed to PAMPs. The binding of triacyl and diacyl lipoproteins and lipopeptides leads to the formation of heterodimers of TLR2 with TLR1 and TLR6, respectively[3,4], and bacterial flagellin induces the formation of TLR5 homodimers[5]. In the case of triacyl lipopeptides, two acyl chains insert into the hydrophobic core of TLR2

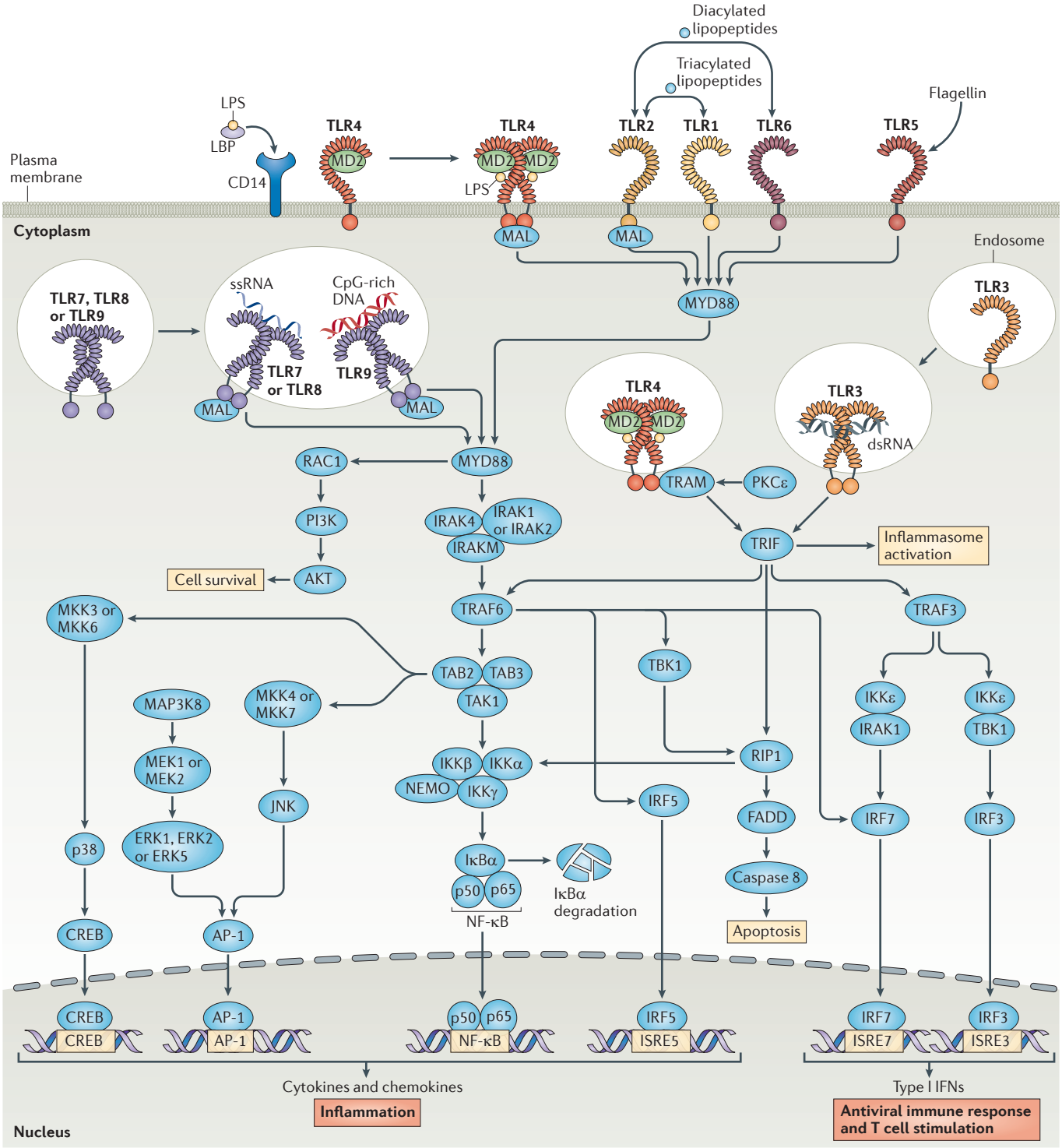

Figure 1 | **Overview of TLR signalling pathways.** Toll-like receptors (TLRs) are present on the cell surface and in endosomes, where they detect microbial cell-wall components, non-self nucleic acids or danger-associated self molecules. Upon stimulation, TLRs activate two types of pathway that involve myeloid differentiation primary response protein 88 (MYD88) and/or TIR domain-containing adaptor protein inducing IFNβ (TRIF). Crosstalk with other signalling pathways ensures that the TLR signal is properly regulated and leads to either apoptosis or cell survival, and the transcription of pro-inflammatory cytokines and chemokines, and type I interferons (IFNs). AP-1, activator protein 1; CREB, cAMP-responsive element-binding protein; dsDNA, double-stranded DNA; dsRNA, double-stranded RNA; ERK, extracellular signal-regulated kinase; FADD, FAS-associated death domain; IκBα, inhibitor of NF-κBα; IKK, inhibitor of NF-κB kinase; IRAK, interleukin-1 receptor-associated kinase; IRF, IFN-regulatory factor; ISRE, IFN-stimulated response element; JNK, JUN N-terminal kinase; LBP, LPS-binding protein; LPS, lipopolysaccharide; MAL, MYD88 adaptor-like protein; MAP3K, mitogen-activated protein kinase kinase kinase 8; MD2, myeloid differentiation factor 2; MEK, mitogen-activated protein kinase/ERK kinase; MKK, mitogen-activated protein kinase kinase; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-κB; PI3K, phosphoinositide 3-kinase; PKCε, protein kinase Cε; RIP1, receptor-interacting protein 1; ssRNA, single-stranded RNA; TAB, TAK1-binding protein; TAK1, TGFβ-activated kinase 1 (also known as MAP3K7); TBK1, TANK-binding kinase 1; TRAF, tumour necrosis factor receptor-associated factor; TRAM, TRIF-related adaptor molecule.

and the third chain binds to a hydrophobic groove on the surface of TLR1, promoting the formation of an extensive protein–protein interface. Flagellin binds directly to the lateral surfaces of TLR5 in a symmetrical arrangement, leading to the formation of a 2/2 complex. By contrast, TLR4 is activated by lipid A — the biologically active constituent of lipopolysaccharide (LPS) — by a more complicated mechanism (FIG. 2a). The recognition of LPS requires the TLR4 co-receptor myeloid differentiation factor 2 (MD2; also known as LY96). MD2 has a β-sandwich structure that provides a hydrophobic core within which the lipid A acyl chains can be accommodated[6,7]. The TLR4 ectodomain forms a rigid curved solenoid with MD2 bound at two conserved sites at the amino terminus. Structural analysis shows that the antagonist eritoran binds to MD2 but is not able to induce a signal. The four acyl chains of eritoran are fully accommodated within the MD2 structure and the diglucosamine backbones are exposed to solvent[8]. By contrast, a high-resolution structure for MD2–TLR4 bound to immunoactive, hexa-acyl lipid A reveals a heterotetrameric complex of MD2 and TLR4 (REF. 9). Hexa-acylated lipid A induces a localized conformational change in MD2 and, as a result, the acyl chain at position 2 is exposed on the surface of the MD2 structure. Together with the MD2 residue Phe126, this creates a hydrophobic patch that forms the dimerization interface with TLR4. Compared to eritoran, the glucosamine backbone of the hexa-acyl lipid A is moved upwards, which repositions the phosphate groups to contact the positively charged residues of both TLR4 subunits and provides further stabilization of the active complex. These initial MD2–TLR4 interactions induce a second dimerization interface between the lateral surfaces of the two ectodomains — an area of extensive protein–protein interaction that is centred on the LRR16 of TLR4 (REF. 9) — and bring the carboxyl termini of the two ectodomains into close proximity to initiate signalling.

Conformational rearrangement of preformed receptor dimers by non-self nucleic acids and small-molecule immunomodulators. TLR7, TLR8 and TLR9 localize to and signal from acidified compartments of the endolysosomal pathway. TLR9 is activated by DNA with unmethylated CpG dinucleotides, and TLR7 and TLR8 respond to single-stranded RNA. TLR7 and TLR8 are also activated by imidazoquinolines and other small synthetic immunomodulatory compounds[10]. One of these molecules, imiquimod, is now in widespread use for the treatment of human papillomavirus infection and basal cell carcinoma. Members of the endosomal TLR subfamily (TLR7–TLR9) also differ from the cell-surface TLRs because they are synthesized as stable preformed dimers[11,12]. In the case of TLR9, this was shown by fluorescence resonance energy transfer techniques. These experiments found that binding of a CpG-containing DNA ligand resulted in a large conformational change in the dimer that was predicted to bring the receptor TIR domains into close proximity. More recently, crystal structures of a TLR8 dimer in an inactive conformation and in complex with imidazoquinoline and thiazoquinoline agonists confirmed this mechanism (FIG. 2b). In the inactive dimer, the lateral surfaces of the LRR solenoids form an extensive interface that is similar to the homodimerization interface seen in TLR4. A molecule of imidazoquinoline or thiazoquinoline (ligands that have a molecular mass of approximately 200 daltons only) binds to a hydrophobic pocket at LRR11 in each protomer, and is oriented and stabilized by stacking interactions with conserved TLR aromatic residues (Phe405 and Tyr348). Ligand binding induces a large conformational change in the TLR dimer interface and causes the two ectodomain protomers to rotate with respect to each other, enabling the bound ligand to make a strong polar interaction with aspartic acid residue 543 located in LRR17. This residue is conserved in the TLR7–TLR9 subfamily and is essential for signalling in response to CpG-containing DNAs, RNAs and azoquinolines[13,14]. The rearrangement of the dimer interface also causes the two ectodomains to tilt together and this brings the juxtamembrane C termini into close proximity.

A key question arising from this remarkable structural study is why both the natural ligands and the synthetic drug molecules require an acidic pH in order to signal. This can be explained in the case of the azoquinolines by the fact that they are cell-permeable weak bases with pK_a values of approximately 7. This means that the molecule will develop a positive charge at pH 5 and make a strong electrostatic bond with Asp543, and this will stabilize the activated conformation. As yet, there is no structure of the TLR8 dimer in complex with RNA and it is not clear which chemical groups in nucleic acids might act as an equivalent weak base. One possibility is the N3 imino group of cytosine, but in the free nucleotide this has a pK_a of 4.2 and would thus remain largely uncharged at the pH in the endosome. However, a recent study has shown that the N3 imino group has a pK_a of 7 when forming a Hoogsteen base pair with guanosine[15]. Interestingly, both ss40 (a TLR8 agonist derived from HIV RNA) and activating CpG-containing oligonucleotides have regions of potential secondary structure that form short G–C duplexes[16]. These regions could flip from a Watson–Crick to a Hoogsteen conformation at pH 5, allowing the protonated cytosine N3 group to make a strong electrostatic bond with the critical Asp543 in TLR8 (Asp534 in TLR9). An arginine residue (Arg429 in TLR8 and Arg426 in TLR9) in the TLR ligand-binding pocket is required for nucleic acid ligands but is dispensable for the small-molecule agonists. This positively charged residue might interact with the phosphate backbone of RNA ligands.

Another distinct characteristic of the TLR7–TLR9 subfamily is that the ectodomains can be cleaved by acid-activated cathepsin and asparagine endoproteases in the endolysosome[17–19]. After cleavage, the N and C termini remain associated and proteolysis may be necessary to prime the receptor for activation by nucleic acids. This processing may provide further protection from the self nucleic acid-induced activation of the receptors that can lead to autoimmune responses.

Protomer
A structural unit of an oligomeric protein. It can be a protein subunit or several different subunits that assemble in a defined stoichiometry to form an oligomer. The protomer is the smallest subset of the different subunits that form the oligomer.

Hoogsteen base pair
An alternative configuration for G–C base pairs in double-stranded nucleic acids. The guanosine base flips around the *N*-glycosidic bond from the *anti* to the *syn* configuration, allowing the formation of a hydrogen bond between the N7 of guanosine and the N3 of cytosine, instead of the N1–N3 hydrogen bond that is found in Watson–Crick base pairs.

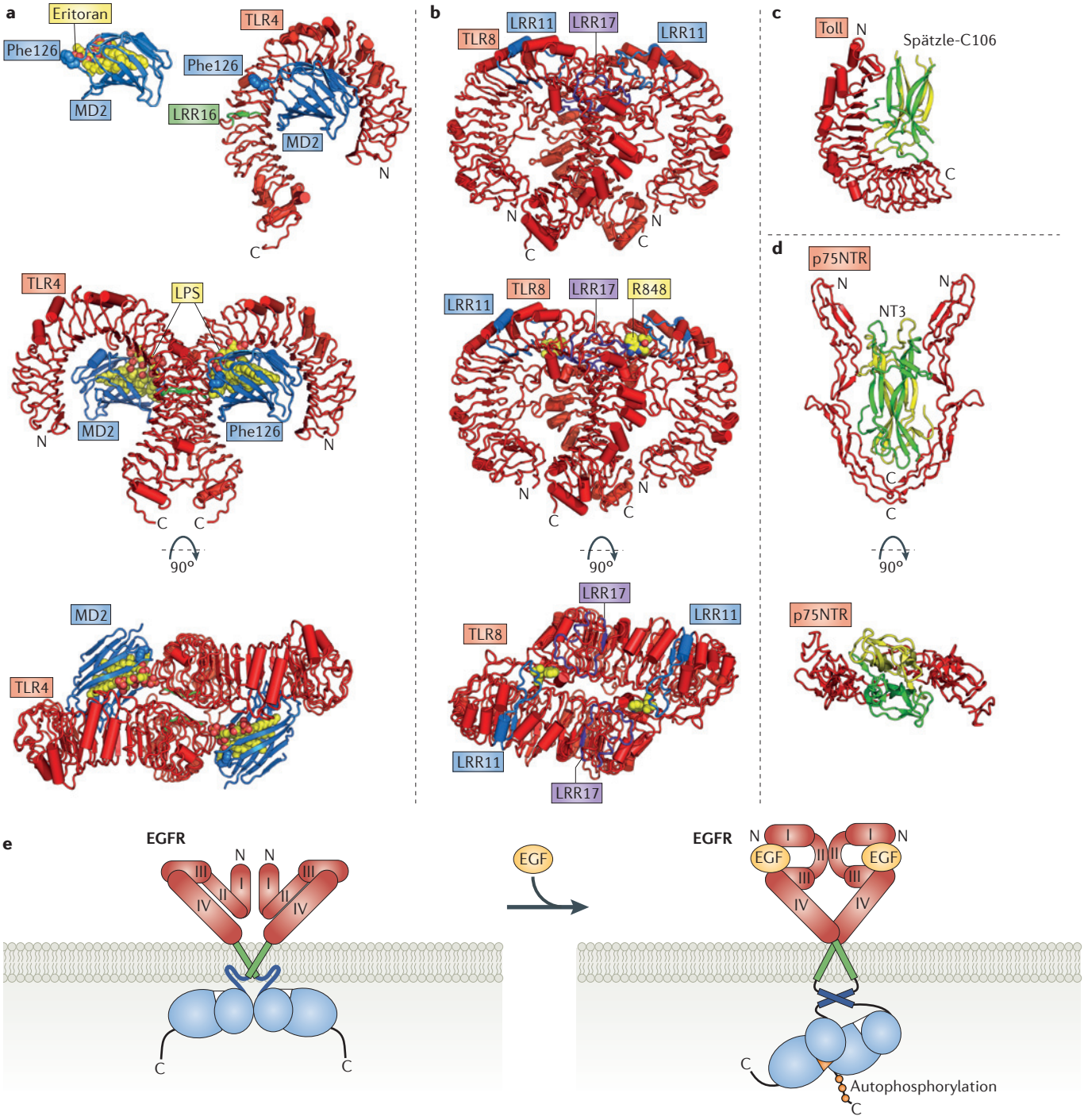

Figure 2 | **Ligand recognition and signal transduction.** **a** | The Toll-like receptor 4 (TLR4) co-receptor myeloid differentiation factor 2 (MD2; shown here bound to the antagonist eritoran, which is shown in yellow and red) and the TLR4–MD2 complex are monomeric in the absence of agonist ligand. In its inactive form, MD2 Phe126 is exposed to the solvent. The binding of lipopolysaccharide (LPS; derived from *Escherichia coli*; shown in yellow and red) triggers a conformational change of the Phe126 loop that forms part of the dimeric interface that is centred on leucine-rich repeat 16 (LRR16; shown in green) together with one of the acyl chains of LPS. Side and top views are shown for the active receptor dimer. **b** | The TLR8 ectodomain is a constitutive dimer. Upon binding of the agonist imidazoquinoline R848 (shown in yellow and red; 3M Pharmaceuticals; also known as resiquimod), the dimer reorganizes in a way that brings the juxtamembrane regions closer together. The ligand-mediated interface is centred around LRR11 (blue) and LRR17 (purple). In parts **a** and **b**, eritoran, LPS and R848 are shown in a sphere representation according to their chemical composition (carbon is shown in yellow and oxygen in red). **c** | The Toll–Spätzle-C106 complex has similarities to the neurotrophin 3 (NT3)–neurotrophin receptor p75 (p75NTR) complex. Spätzle-C106 forms extensive asymmetric contacts with the concave side of Toll. Spätzle-C106 is a covalent dimer (shown in green and yellow). **d** | The binding of dimeric NT3 to its receptor p75NTR triggers crosslinking of two receptor chains in a symmetrical complex. **e** | Epidermal growth factor receptor (EGFR) forms an inactive dimer that undergoes conformational rearrangements following ligand binding. Conformational changes induced by ligand binding release steric constraints and reposition the transmembrane helices (shown in green) so that they can interact at their amino termini. In turn, the intracellular juxtamembrane regions (shown in blue) adopt an antiparallel conformation, which releases the inhibition of the kinase domain by the membrane.

TLR3 also signals mainly from acidified compartments but, in contrast to TLR7–TLR9, it is a monomer when inactive. TLR3 is directly crosslinked by double-stranded RNA. pH-dependent activation is conferred by conserved histidine residues that become protonated and make crucial contacts with the TLR3 ectodomain[20]. Activation is also accompanied by the lateral clustering of the receptors (see below)[21].

Indirect coupling of Spätzle ligand binding and dimerization of* D. melanogaster *Toll. Unlike the TLRs, *D. melanogaster* Toll is activated by the endogenous cytokine-like ligand Spätzle. Spätzle is secreted in an inactive form and proteolytically activated to form dimeric Spätzle-C106 (the 106 C-terminal amino acids of inactive Spätzle) that is structurally similar to vertebrate neurotrophins. The crystal structure of the Toll–C106 complex reveals a 1/1 complex with a binding mode that is reminiscent of mammalian neurotrophins, such as neurotrophin 3 binding to neurotrophin receptor p75 (also known as TNFRSF16) (FIG. 2c,d). The covalent C106 dimer forms asymmetric contacts at the concave side of the N-terminal cap and within the first ten LRRs[22]. In contrast to TLRs, the ligand does not induce dimerization of the receptor in the crystal structure but biochemical evidence suggests that the active complex is a heterotetramer with two molecules of receptor and two molecules of Spätzle-C106 (REF. 23). Although a structure of this active 2/2 complex has not been solved, it is likely that allosteric interactions are involved in Toll signalling, whereby ligand binding to the N terminus induces a conformational change that promotes the homodimerization of juxtamembrane regions in the Toll ectodomain C termini.

Signal transduction

Irrespective of the mode of dimerization, activated Toll and TLRs bring the juxtamembrane sequences at the C terminus of the two ectodomains into close proximity. These juxtamembrane modules consist of an antiparallel β-sheet that is stabilized by two disulphide bonds and they are connected to the transmembrane helix by a very short linker (about three amino acids in length)[24]. In the case of Toll, but not the TLRs, mutation of any of the four cysteine residues in this capping structure causes constitutive activation, potentially owing to the release of steric hindrance that is conferred by the cap that prevents receptor multimerization. Similarly, severe truncation of the Toll and TLR ectodomains leads to constitutive receptor signalling, which suggests that the ectodomains are autoinhibitory and that ligand binding relieves this inhibition[25]. Thus, the juxtamembrane and transmembrane domains of the TLRs have an intrinsic propensity to dimerize.

At present, little is known about the conformational changes that occur in the transmembrane α-helices to promote TIR domain dimerization. Nevertheless, it is likely that TLR activation has features in common with other type 1 receptors, particularly the epidermal growth factor receptor (EGFR). In the inactive state, the transmembrane helices of EGFR interact at their C-terminal ends, which is consistent with the juxtamembrane sequences of the ectodomains having an autoinhibitory effect, as is observed with the TLRs (FIG. 2e). Constitutive and ligand-induced activation leads to a repositioning of the transmembrane helices to form a new intermolecular dimerization interface at the N terminus. This causes the transmembrane helices to be oriented at an angle of approximately 45 degrees and, as a result, the C-terminal ends become separated from each other by approximately 20 Å. Besides hydrophobicity, there is little sequence requirement for the transmembrane helices, although the N-terminal dimerization interface has a preference for amino acids with small side chains[26].

The cytosolic juxtamembrane sequences of the TLRs link the transmembrane helices to the TIR domains and are rather diverse, varying in length from 17 amino acids in TLR4 to 28 in TLR1, TLR6 and TLR10 (REF. 24). They tend to be basic in character and are strongly predicted to form an α-helical secondary structure. In EGFR, the corresponding sequences are also basic and, in the inactive conformation, they are sequestered in the membrane by interacting with anionic phospholipid head groups. During receptor activation, the juxtamembrane sequences of two EGFR molecules are pulled off the plasma membrane and they reassemble as antiparallel α-helices. This allows the formation of an asymmetrical kinase dimer and cross-phosphorylation (FIG. 2e). Although TIR domains do not have kinase activity, it is likely that a similar process occurs with the TLRs, causing the TIR domains to associate in the correct configuration for the recruitment of downstream signal transducers.

Post-receptor complex assembly: TIR domains

Structure and cellular localization. There are five TIR domain-containing signalling adaptor proteins that mediate signal transduction by the TLRs. MYD88 is required by all TLRs except for TLR3, which uses TRIF alone. TLR4 signals through both the MYD88- and TRIF-mediated pathways, which involve the bridging adaptor proteins MYD88 adaptor-like protein (MAL; also known as TIRAP) and TRIF-related adaptor molecule (TRAM; also known as TICAM2), respectively[27] (FIG. 1). Structures have now been determined for both receptor and adaptor TIR domains[28–33] (FIG. 3). They have a common α/β-fold with a core of four or five parallel β-strands (referred to as βA–βE strands) that are surrounded by five α-helices (αA–αE helices) (FIG. 3c). The loops that connect these secondary structures have a central role in signal transduction, especially the BB loop that connects the βB strand to the αB helix. A variant form of TLR4 that has a single amino acid change in the BB loop (Pro712His in mice, which corresponds to Pro714 in humans) is unresponsive to LPS[34] and is dominant negative. The BB loop is crucial for the function of most, if not all, receptor and adaptor TIR domains. The adaptor protein MAL has a different topology compared with other TIR domain-containing proteins, as it is missing the αB helix. Instead, the αA helix is connected directly

Allosteric interactions
Interactions between two topographically distinct binding sites on the same receptor complex. These interactions can be between two ligand binding sites or between a ligand binding site and an effector binding site.

to the βB strand and βC strand, and thus contains a long loop (AB loop) that connects the first helix (αA helix) and the βB strand. The MAL AB loop nevertheless retains BB loop features and is important for adaptor function.

In resting cells, MYD88 and TRIF seem to be dispersed throughout the cytosol[35], although in some cell types MYD88 is observed as a punctate inclusion, which reflects the propensity for this adaptor to assemble homo-oligomers[36]. By contrast, the bridging adaptor proteins

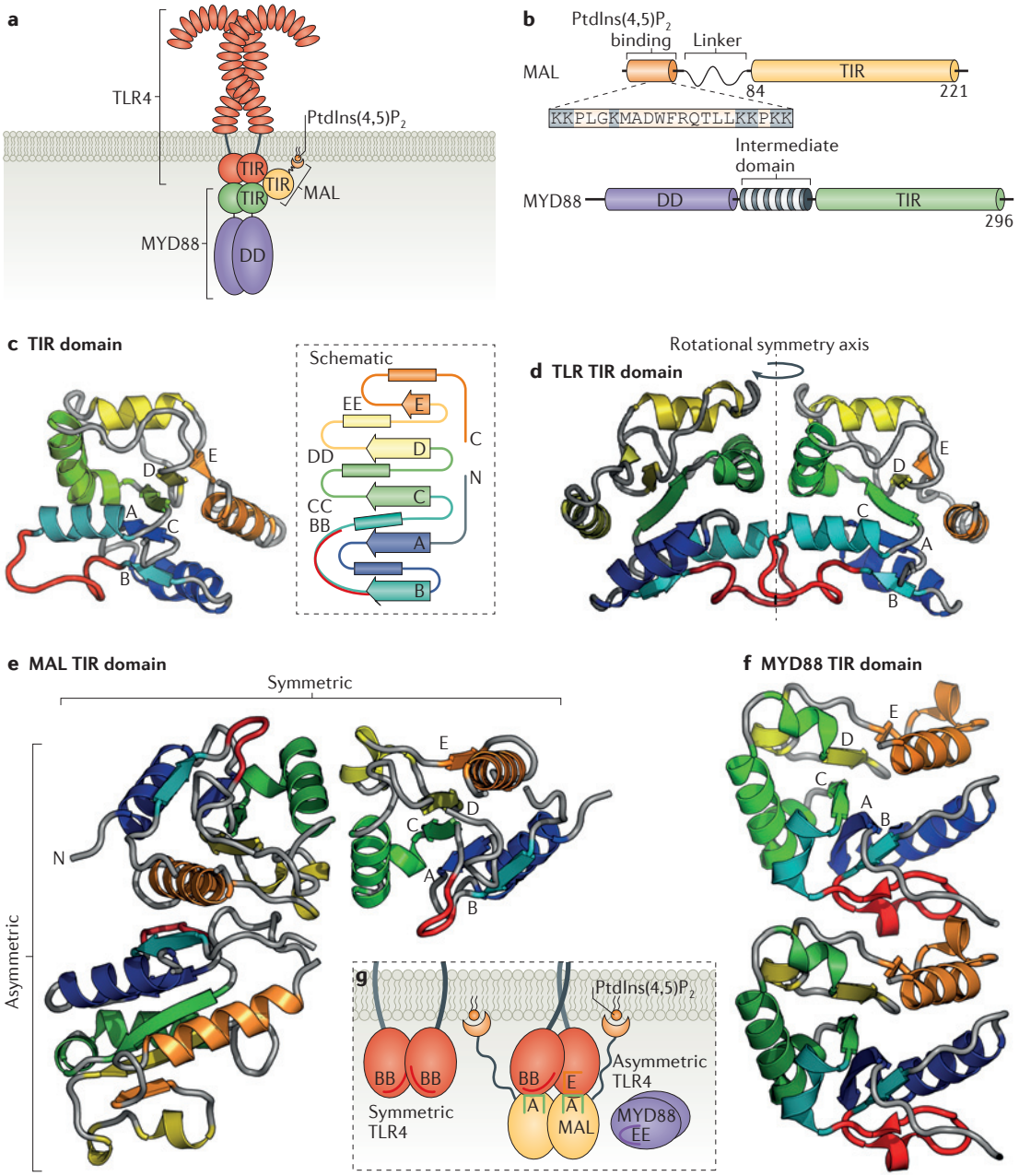

Figure 3 | **Interactions underlying signalling through TIR domain-containing complexes.** **a** | A schematic showing the assembly of the adaptor proteins myeloid differentiation primary response protein 88 (MYD88) and MYD88 adaptor-like protein (MAL) with Toll-like receptor 4 (TLR4) through their Toll/IL-1R (TIR) domains. **b** | The adaptor protein TIR domains are linked to amino-terminal domains that contain a phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$)-binding motif in MAL (sequence boxed with positively-charged lysine residues highlighted in blue) and a death domain (DD) in MYD88. **c** | The TIR domain has a central parallel five-stranded β-sheet and flanking α-helices, shown here as a three-dimensional structure with a two-dimensional schematic for clarity. **d** | Structures of TLR TIR domains have revealed a dimer with a rotational symmetry axis — shown here for TLR10. **e** | The structure of the MAL TIR domain revealed a similar symmetric association (viewed along the symmetry axis here) and an asymmetric association, involving the E helix (orange). **f** | The MYD88 TIR domain in isolation has been observed to form head-to-tail dimers. **g** | The multiple interactions that are made between TIR domain dimers that are involved in TLR4 signalling are shown schematically.

MAL and TRAM are localized to the cytosolic surface of the plasma membrane by different mechanisms. In the case of MAL, a basic motif at the N terminus preceding the TIR domain binds to the head group of the lipid phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$)[37] (FIG. 3b). By contrast, TRAM is co-translationally modified by the addition of a myristoyl group to a glycine residue at position 2 (REF. 38). This fatty acyl chain partitions into the membrane, and the association of TRAM with the membrane is stabilized by electrostatic interactions between adjacent basic residues and phospholipid head groups. The phosphorylation of serine residues within this basic motif by protein kinase Cε disrupts this interaction and releases TRAM from the membrane. This myristoyl–electrostatic switch is necessary for robust activation of the TRAM–TRIF pathway by TLR4 (REF. 39).

Molecular basis of TIR domain–TIR domain interactions. In contrast to the monomeric receptor and adaptor protein TIR domains, structures of homo-oligomers and hetero-oligomers of TIR domain-containing proteins have proved elusive. Typically, purified TIR domains do not form stable complexes *in vitro*. Nevertheless, experiments using mutagenesis, cell-permeable inhibitory peptides, molecular docking and crystallographic analysis have revealed possible arrangements for some receptor and adaptor protein TIR domains in post-receptor complexes.

A putative homodimer of the TLR4 TIR domains was modelled using a symmetrical dimer that was observed in the crystal structure of the TLR10 TIR domain (FIG. 3d). In the predicted homodimer, the interface is extensive and involves the BB loops of both subunits. The conserved proline residue confers the BB loop with a rigid conformation and substitution with other residues would cause a marked distortion in the geometry of the homodimer interface[40]. Another important conclusion of this study is that the receptor TIR domains associate with a twofold axis of symmetry, such that the juxtamembrane linkers would be oriented on the same surface[40] (see above). In this regard, it is interesting that a flat, but slightly curved, surface is predicted to form the membrane-proximal surface. This feature is seen in other proteins that interact with membrane surfaces, such as the BAR domain of amphiphysin[112]. Molecular docking of MAL and TRAM to TLR4 suggests that the two adaptor molecules bind to symmetry-related sites at the homodimer interface. The properties of inhibitory peptides that are derived from the TLR4 BB loop and the small-molecule antagonist TAK-242 (also known as resatorvid; from Takeda Pharmaceutical) — a thiol reagent that reacts specifically with cysteine residues in the dimer interface — are consistent with this arrangement of the post-receptor complex, as they block signalling that is mediated by both MAL and TRAM[41,42]. TRAM is recruited to TLR4 only after endocytosis of the activated complex (FIG. 1). A possible explanation for this is that the dimerized receptor ectodomains undergo a rearrangement in the acidic environment owing to the protonation of histidine residues in the homodimer interface. This could cause a conformational change, leading to an arrangement of the TIR domain dimer that is unique to TRAM[43].

MAL is of particular interest because two human single nucleotide polymorphisms (SNPs) that encode Ser180Leu and Asp96Asn variants of MAL confer susceptibility to infectious diseases, including tuberculosis[44,45]. The MAL TIR domain is monomeric in solution but probably functions as a dimer *in vivo*. In the crystal structure, a potential homodimer with a twofold axis of symmetry is observed and it was confirmed as functionally important by mutagenesis (FIG. 3e). The dimerization interface involves hydrophobic interactions between the αC helices and, in this configuration, the N termini have the same orientation, such that both subunits would be able to bind to $PtdIns(4,5)P_2$ in the membrane, thus stabilizing the assembly[31]. It is unclear whether the binding of MAL to the activated receptor causes the dimer to break and dissociate from the membrane in a cooperative assembly process. The two disease-associated SNPs cause the substitution of amino acids that are located close together on the surface of MAL and that are predicted to form an acidic binding site for MYD88. In particular, an arginine residue (Arg196) in the BB loop of MYD88 forms an electrostatic interaction with Asp96 in wild-type MAL. Interestingly, individuals who are homozygous for the Arg196Cys allele are defective in MYD88-mediated signalling and are highly susceptible to infection by Gram-positive bacteria[46].

Several other potential arrangements for TIR domain–TIR domain complexes have been proposed. Mice that are homozygous for a mutation in the MYD88 TIR domain, known as Pococurante, lack signalling by most TLRs, although the activation of TLR2–TLR6 by diacyl lipids is unaffected[47]. The mutation changes an isoleucine in the αA helix to an asparagine and Pococurante MYD88 cannot be recruited to activated receptors other than TLR2–TLR6. Together with evidence from binding studies, this suggests an antiparallel 'head-to-head' interaction between the receptor and adaptor protein TIR domains that is mediated by the αA helices, which perhaps stabilizes a primary interface involving the BB loops. This study also identified two conserved aromatic residues in the αE helix that have important roles in signalling. These sites in MYD88 are required for distal signalling but not for its recruitment to TLR2. The importance of the αE helix for MYD88 function is also demonstrated by studies using inhibitory peptides derived from TLR4. Only peptides that are derived from the juxtamembrane linker, the BB loop and αE helix are able to both inhibit signalling and bind to the TLR4 TIR domain[48]. These results suggest that activated TLR4 may be an asymmetric homodimer with an interface that is formed from the BB loop of one subunit and the αE helix of the other (FIG. 3g). Studies with peptide inhibitors also suggest that the TRIF BB loop binds to TLR4 but not to TRAM, whereas the αB helix associates strongly with TRAM and only weakly with TLR4 (REF. 49). However, another study has identified residues on the αE helix and EE loop of TRIF that are required to form the interface with a symmetric TRAM homodimer, and has shown that this is arranged in a similar way to that of TLR homodimers[33].

The subtle sequence requirements for adaptor protein specificity are illustrated by a recent study of TLR3 (REF. 50). Uniquely among the human TLRs, TLR3 has an alanine rather than a proline in the BB loop. Remarkably, mutation of this one residue in TLR3 to proline causes a switch in adaptor protein specificity from TRIF to MYD88 (REF. 50). In cells expressing the proline TLR3 mutant, IRF3-dependent responses are abolished and the activation of NF-κB is substantially enhanced. These studies also found that both TRIF and MYD88 are associated with TLR3 before stimulation, either in a direct complex or as part of detergent-rich membrane domains (see below).

Overall, the current evidence suggests that bimolecular interactions between TIR domains are weak and that ternary TIR domain–TIR domain complexes are stabilized by multiple types of interaction. Entropic effects that arise from the membrane localization of receptors and adaptor proteins may also contribute to the assembly of the complexes during signal transduction. It is also likely that the assembly process is allosteric in nature.

Higher-order scaffolds

Importance of positive and negative allostery. For TLR4, TLR3 and TLR9, there is evidence that receptor activation occurs within a range of ligand concentrations that are within an order of magnitude, and this is consistent with positive cooperative binding[12,51,52]. By contrast, *D. melanogaster* Toll signalling is induced by a large range of ligand concentrations in a cell-based assay and this is a property of a negative cooperative binding[53] (see Supplementary Information S1 (figure)). These substantial mechanistic differences are reflected in the distinct ligand binding modes described above. The assembly of intracellular signalling scaffolds, such as the Myddosome, may also contribute to the positive allostery that is displayed by the TLRs. By contrast, simpler, linear complexes of adaptor proteins mediate *D. melanogaster* Toll signalling. Allosteric interactions in the Myddosome may require the helical assembly of the adaptor protein subunits and therefore Toll may not have analogous cooperativity.

Helical assembly of MYD88 and IL-1R-associated kinases: the Myddosome. The MYD88 adaptor protein is a modular protein with a death domain (DD) that is connected to the TIR domain by a linker known as the intermediate domain (FIG. 3b). DDs are found in a family of about 40 signal transducers that include receptors and adaptor proteins of the FAS (also known as TNFRSF6)–FAS-associated death domain (FADD) apoptotic signalling pathway[54]. DDs have a structure consisting of six antiparallel α-helices that are arranged in a Greek key motif (see the inset of FIG. 4d). This topology is shared with other signal transducers that are involved in innate immunity and apoptosis, such as those with death effector domains, caspase recruitment domains and pyrin domains. In contrast to TIR domains, DDs can form stable homo-oligomers and hetero-oligomers. Structural analysis has defined three discrete modes by which DDs can associate to form a variety of complexes — these are known as type 1, 2 and 3 interactions (FIG. 4c,d). During signal transduction by the TLR and IL-1R families, MYD88 assembles with the DD-containing IL-1R-associated kinase (IRAK) family. In vertebrates, there are four IRAK paralogues (IRAK1, IRAK2, IRAKM (also known as IRAK3) and IRAK4) (FIG. 4b), whereas insects have a single gene, *Pelle*, which encodes a kinase that is most similar to IRAK4 (REF. 55).

In the absence of a stimulus, cytosolic MYD88 is in a repressed state, although overexpression of the full-length protein or the DD (but not the TIR domain) causes constitutive activation of the pathway[56]. It is possible that MYD88 is kept in an autoinhibited conformation by an intramolecular interaction between the DD and the TIR domain, and that this is disrupted when the TIR domain assembles in a post-receptor complex[57]. *In vitro*, the MYD88 DD forms a heterogeneous mixture of dimers and higher-order oligomers but in the presence of IRAK4, these assemble into a discrete heterocomplex — the Myddosome[52]. The MYD88–IRAK4 Myddosome has a variable stoichiometry, with six to eight molecules of MYD88 to four molecules of IRAK4, and it may also form smaller sub-complexes. A crystal structure of a variant Myddosome containing IRAK2, as well as IRAK4, has revealed a remarkable hierarchical arrangement of the subunits[58]. The complex consists of three layers with six MYD88 DDs, four IRAK4 DDs and four IRAK2 DDs that are arranged as a left-handed helix, with 3.7 subunits per turn (FIG. 4e). Importantly, the helix is stabilized specifically by type 3 DD–DD interactions, suggesting a sequential assembly process in which homo-oligomers of MYD88 preferentially recruit four IRAK4 DDs and then four IRAK2 DDs. This is characteristic of a process that displays positive cooperativity. However, the molecular basis of this allosteric interaction is unclear and it does not seem to involve large protein conformational changes, as the homotypic type 3 interactions between the MYD88 subunits are structurally equivalent to the heterotypic interaction between the sixth MYD88 and the first IRAK4 subunit. Alternatively, the mechanism might involve dynamically driven allosteric interactions in which constraints on the mobility of MYD88 interface residues in the homo-oligomer confer specificity for association with the first IRAK4 subunit[59].

Complex assembly as a prerequisite for downstream signal transduction. A key question regarding the Myddosome is whether the complex is physiologically important for signal transduction or whether it is an *in vitro* artefact. In that regard, the solved structure used MYD88 that lacks the intermediate domain, a linker that connects the DD and TIR domain and that is required for signalling function[60,61]. This suggests that additional intermediate domain–DD interactions that are not defined in the currently available Myddosome structure are essential for signalling *in vivo*. On the other hand, a naturally occurring SNP that changes serine 34 of the MYD88 DD to tyrosine is defective for signalling *in vivo* and for Myddosome

Positive cooperative binding
Cooperative binding occurs if the number of binding sites of a receptor that are occupied by ligand is a nonlinear function of ligand concentration. Positive cooperative binding of a ligand increases the apparent affinity of the receptor (for example, by inducing a conformational change) and hence increases the chance of another ligand molecule binding. The presence of preformed Toll-like receptor 8 dimers in the absence of single-stranded RNA is an example of positive cooperative binding.

Negative cooperative binding
A form of interaction that involves the binding of a ligand that decreases receptor affinity and hence makes the binding of other ligand molecules less likely. The presence of ligand-bound Toll–Spätzle monomers demonstrates negative cooperative binding.

Greek key motif
A common structural motif that consists of four adjacent antiparallel strands and their linking loops. Three antiparallel strands are connected by hairpins, whereas the fourth is adjacent to the first and is linked to the third by a longer loop.

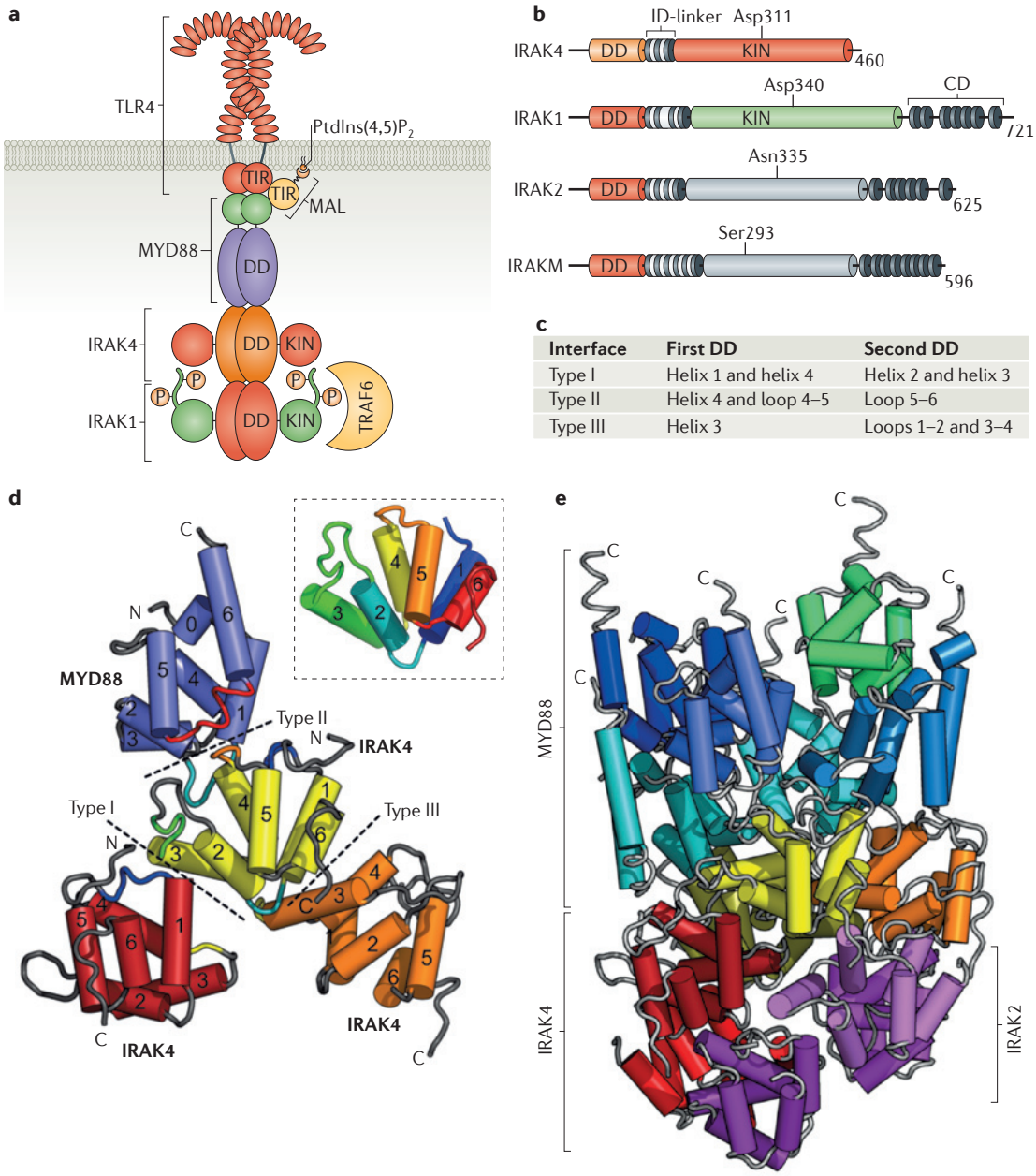

Figure 4 | **Death domain interactions position protein kinases in the Myddosome assembly.** **a** | The arrangement of adaptor protein death domains (DDs) and kinase (KIN) domains in association with the Toll/IL-1R (TIR) domains of Toll-like receptor 4 (TLR4) is shown here schematically. **b** | The domains of IL-1R-associated kinase (IRAK) family proteins are shown. These include the DD, the intermediate domain (ID), the KIN domain and the carboxy-terminal domain (CD). The key catalytic aspartate residues that are present in IRAK4 and IRAK1 (Asp311 and Asp340, respectively) are mutated in the inactive kinases IRAK2 and IRAKM (Asn335 and Ser293, respectively). **c** | The interfaces that are involved in DD assembly are listed. **d** | DDs involved in myeloid differentiation primary response protein 88 (MYD88) and IRAK4 contacts in the Myddosome. The inset shows an equivalent view of the IRAK4 DD with helices in rainbow colours. **e** | The complete Myddosome DD assembly is shown with MYD88 DDs in blues and green, IRAK4 DDs red, orange and yellow, and IRAK2 DDs in violets. MAL, MYD88 adaptor-like protein; PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; TRAF6, tumour necrosis factor receptor-associated factor 6.

formation *in vitro*[62]. Molecular modelling indicates that a bulky tyrosine residue at this position would sterically interfere with the type 3 DD–DD interactions that drive helical assembly, which provides evidence that the helical complex is required for function. A recent study of the paralogue IRAKM also supports the physiological importance of Myddosomes[63]. This work shows that, in the absence of IRAK1 and IRAK2, IRAKM can assemble with MYD88–IRAK4 and mediate the 'second wave' activation of NF-κB downstream of TLR7 in primary macrophages. By contrast, IRAKM inhibits translational regulation of cytokine and chemokine production in the

presence of IRAK2, and this raises the possibility that in these conditions it can form a fourth Myddosome layer[63]. Thus, the composition of Myddosome complexes and the precise signalling output will depend on the cellular context.

Myddosome complexes and human disease. The importance of the Myddosome for host defence is illustrated by the identification of a patient who has a mutation in the IRAK4 DD that changes arginine 12 to cysteine (Arg12Cys) and who is a compound heterozygote with a loss-of-function frameshift allele[64]. The patient has a history of severe infections by pyogenic bacteria and has completely defective MYD88-dependent cytokine responses. In the Myddosome structure, Arg12 contributes to a crucial type 2 DD–DD interface with MYD88 residues Asp100 and Leu103. Importantly, a recent report shows that the Arg12Cys IRAK4 mutant cannot signal to NF-κB or assemble into a Myddosome[65]. Arg12 is highly conserved in vertebrate IRAK4 proteins but is not present in the other IRAK paralogues. It is, however, found in the *D. melanogaster* IRAK4 homologue Pelle. Activation of the *D. melanogaster* Toll pathway leads to the formation of a simpler heterotrimeric complex of MYD88 with the adaptor proteins Tube and Pelle that is topologically equivalent to a segment of the Myddosome structure[58,66] (FIG. 4). The Pelle residue Arg35, which is equivalent to IRAK4 Arg12, interacts with Tube Glu50 in a type 2 DD–DD interface[67]. Neither the Pelle mutant Arg35Glu, nor the Tube mutant Glu50Lys, is active but strikingly, when the two mutations (Pelle Arg35Glu and Tube Glu50Lys) are expressed together, high levels of Toll signalling are restored. This shows that fundamental aspects of Toll signalling are conserved in evolution.

Oncogenically active somatic mutations of MYD88 are found in some types of B cell lymphoma, such as Waldenstrom's macroglobulinaemia and diffuse large B cell lymphoma[68,69]. These mutants of MYD88 are dominant positive and cause constitutive activation of NF-κB, which leads to the sustained production of cytokines and cell survival. A single point mutation in the MYD88 TIR domain, Leu265Pro, is found in the majority of these tumours. The stimulus-independent activity of Leu265Pro MYD88 — which also causes activation of IRAK4 and IRAK1 — can be accounted for by a de-repression of resting MYD88, which drives the constitutive assembly of Myddosomes. So drugs that can interfere with Myddosome assembly may find widespread use not only as anti-inflammatory agents but also as anticancer agents.

The Triffosome in IFNβ-directed signalling. Compared to MYD88, TRIF has a more complex multimodular structure of 712 amino acids. The α-helical N-terminal domain of TRIF (TRIF-NTD) is followed by a proline-rich region with binding sites for the downstream effector proteins tumour necrosis factor (TNF) receptor-associated factor 2 (TRAF2) and TANK-binding kinase 1 (TBK1). The TIR domain and a receptor-interacting protein (RIP) homotypic interaction motif (RHIM) domain constitute the C terminus of the molecule[70–72]. The TRIF-NTD has a structure consisting of eight antiparallel α-helices — a structure that is similar to tetratricopeptide repeat proteins, which mediate protein–protein interactions in the assembly of multiprotein complexes[73]. In unstimulated cells, the TRIF-NTD acts as a negative regulator by binding to the TIR domain and preventing access to the binding sites of downstream effector proteins TRAF3, TRAF6, TBK1 and RIP1 (also known as RIPK1) or RIP3 (also known as RIPK3)[70]. The TRIF TIR domain binds to active TLR3 and TLR4–TRAM, and this releases the TRIF-NTD from the complex, which enables the binding of TBK1 and TRAF3 for activation of IRF3 and/or IRF7. RIP1 binds to the TRIF RHIM domain causing both FADD-dependent apoptosis and the activation of NF-κB by the inhibitor of NF-κB (IκB) kinase complex[71,74]. In some situations, TRAF6 can also bind to TRIF and activate NF-κB[75,76]. At present, the precise composition and stoichiometry of the TRIF signalling complex is unclear. Similar to the formation of the Myddosome by MYD88, TRIF may form higher-order molecular scaffolds. In the resting state, TRIF is diffusely distributed throughout the cytoplasm of a cell but activation of TLR3 and TLR4 or deletion of the TRIF-NTD leads to the formation of large inclusions that also contain the downstream effector proteins[35,70]. This suggests that a cooperative assembly process, analogous to that of the Myddosome, may operate in TRIF signalling.

The signalling pathways mediated by TRIF-containing protein complexes have a crucial role in antiviral host defence. Members of families with autosomal dominant and recessive mutations of *TRIF* are susceptible to childhood encephalitis caused by herpes simplex virus (HSV)[77]. The recessive mutant TRIF proteins are completely defective in mediating signalling through both TLR3 and TLR4. By contrast, the dominant mutation changes a serine to a leucine at the C terminus of the TRIF-NTD close to the TBK1-binding motif and only affects TLR3 signalling. Loss-of-function mutations in TLR3, TRAF3 and UNC93B (also known as UNC93B1) — a chaperone protein of the endosomal TLRs (see next section) — also confer susceptibility to HSV encephalitis[78–80]. Although the observed predisposition to this disease is rather specific, this may be because antiviral innate immunity mediated by TRIF is redundant with the cytosolic pathways that are mediated by retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) in many other viral infections.

The TRIF pathway also has a crucial role in regulating adaptive immunity. It has been shown that activation of TLR3 in mouse $CD8\alpha^+$ myeloid dendritic cells (mDCs) is required for cross-presentation of exogenous antigens in dying, virally infected cells that have been taken up by phagocytosis[81]. This TRIF signal leads to the activation of cytotoxic $CD8^+$ T cells rather than to cross-tolerance. Subsequent studies found that the human DC subset that expresses CD141 (also known as BDCA3 and thrombomodulin) fulfils an analogous role, which emphasizes the importance of the TLR3–TRIF signalling axis for antiviral adaptive immunity in mammals[82]. TRIF signalling in mDCs is also necessary for the activation of natural killer cells, which leads to inhibition of tumour growth in a mouse model[83].

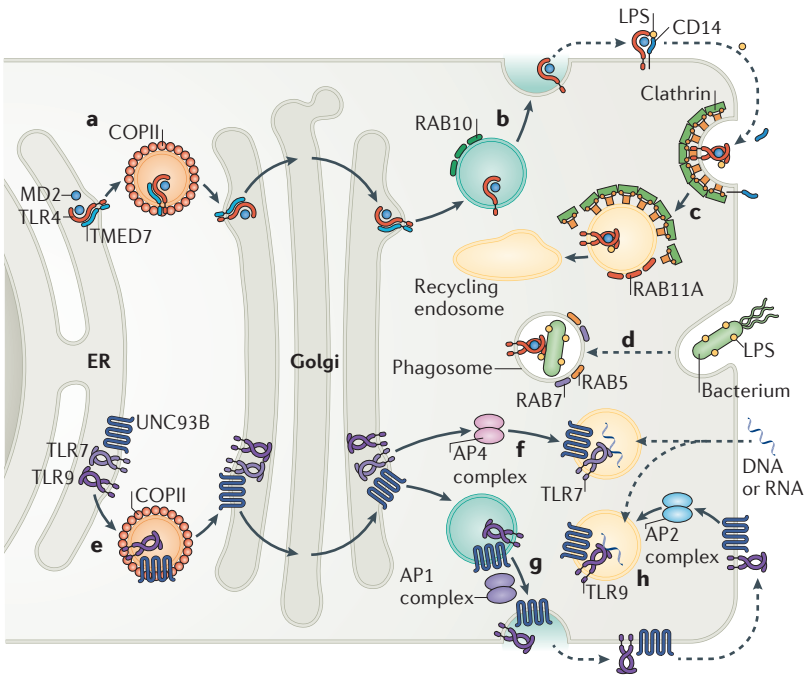

Figure 5 | **Chaperones and pathways in the cellular trafficking of TLRs.** The cellular trafficking pathways of Toll-like receptor 4 (TLR4) are shown in the upper half of the cell with the TLR7 and TLR9 pathways in the lower half. TLR4 associates with its co-receptor myeloid differentiation factor 2 (MD2) and transmembrane emp24 domain-containing protein 7 (TMED7) in the endoplasmic reticulum (ER) (**a**) and is trafficked to the Golgi in a coat protein complex II (COPII)-coated vesicle before export to the plasma membrane in a RAB10-coated vesicle (**b**). The TLR4–MD2 complex associates with lipopolysaccharide (LPS) together with the co-receptor CD14 (which exists as both membrane-bound and soluble forms). This can result in signalling but the complex can also be endocytosed in a clathrin-coated RAB11A-associated vesicle (**c**) for intracellular signalling and recycling. Similar steps are involved in the recognition of LPS as part of the outer membrane of bacteria in phagosomes (**d**). TLR3, TLR7, TLR8 and TLR9 require the chaperone UNC93B for trafficking from the ER in COPII-coated vesicles to the Golgi (**e**). TLR7 is transferred to endosomes by the adaptor protein 4 (AP4) complex, where it can associate with its ligand DNA and RNA nucleic acids (**f**). TLR9 is exported to the plasma membrane (**g**) before being similarly localized to the endosomal membrane by the AP2 complex (**h**). Solid arrows indicate well-defined pathways, whereas dotted lines show putative or incompletely understood pathways.

Cell biology of TLR signalling

Trafficking. TLR pathways are subject to complex regulation that operates not only on the signal transduction process itself but also at the level of biosynthesis, trafficking to the cell surface and endolysosomal compartments, endocytosis and phagocytosis (FIG. 5). The secretion of cell-surface transmembrane proteins is initiated by the translocation and folding of the protein in the endoplasmic reticulum (ER). Proteins that are destined for secretion, rather than ER residence, are then selectively packaged into vesicles for transport to the *cis*-Golgi. In the case of TLR4, the chaperone molecules heat shock protein 90 kDa β1 (HSP90β1; also known as endoplasmin, GRP94, GP96 and TRA1) and protein associated with TLR4 (PRAT4A; also known as CNPY3) are required for proper processing of TLR4 in the ER[84–86]. The association of TLR4 with MD2 in the ER is also crucial for correct glycosylation, secretion to the plasma membrane and, therefore, LPS responsiveness[87–89]. A recent study has found that the secretion of TLR4 also requires transmembrane emp24 domain-containing protein 7 (TMED7), which is an adaptor protein that selects correctly folded cargo in the ER for packaging into coat protein complex II (COPII)-coated vesicles and trafficking to the *cis*-Golgi and cell surface[90]. Another level of regulation is provided by the small G protein RAB10, which controls the rate of TLR4 trafficking from the Golgi to the plasma membrane in response LPS[91]. The stimulation of TLR4 by LPS induces internalization of the receptor by clathrin-mediated endocytosis, a process that also requires the accessory protein CD14, the GTPase RAB11A and, potentially, signalling by spleen tyrosine kinase (SYK)[92–94]. Overall, this suggests that signalling by cell-surface TLRs is a highly dynamic process. So, on the one hand, ligands may induce the rapid internalization of activated receptors and, on the other hand, they may enhance the secretion of the newly synthesized receptors to the surface. Sustained signal transduction may depend on the balance of these processes.

In contrast to TLRs that signal from the cell surface, the endosomal TLR3, TLR7, TLR8 and TLR9 are recognized by another chaperone, UNC93B. UNC93B is an intrinsic membrane protein that is predicted to have 12 transmembrane helices and it is related to the *Caenorhabditis elegans* K^+ channel protein UNC93. A mouse mutant — referred to as 3d — that is highly susceptible to viral infections has a missense mutation that introduces a charged arginine residue into the ninth transmembrane helix of UNC93B and abolishes signalling by all of the endosomal TLRs[95]. Humans with truncated forms of UNC93B have the same sensitivity to HSV encephalitis as humans with TRIF or TLR3 mutations[80]. A recent study has found that, similar to TMED7, UNC93B acts as an adaptor protein for anterograde trafficking to the Golgi, and for packaging TLR7 and TLR9 into COPII-coated vesicles. However, in contrast to TMED7, UNC93B seems to promote onward transport to the endosome in an adaptor protein complex 2 (AP2)- and AP4-dependent manner for TLR9 and TLR7, respectively. Acidic residues in the extracellular juxtamembrane sequences of the endosomal TLRs confer specificity for UNC93B[96].

Microdomains. Membrane microdomains, also known as lipid rafts, are regions of membrane that have a distinct lipid composition, such as high concentrations of cholesterol[97]. Microdomains can act as organizing centres for signalling molecules and are usually associated with the plasma membrane. Although the existence of lipid rafts in live cells has been controversial[98], there is evidence that they regulate signal transduction in several pathways, including the EGFR and the T cell receptor signalling pathways[99,100]. Lipid rafts have a pivotal role in sensitizing and desensitizing signalling by TLRs. On the one hand, cholesterol loading of macrophages or the stabilization of rafts with cholera toxin B strongly enhances the activation of inflammatory signalling[52,101]. On the other hand, the depletion of free cholesterol — either by treatment with cyclodextrins and other raft-disrupting agents or in mice lacking the cholesterol efflux pump ATP-binding cassette subfamily A1 (ABCA1) — leads to the downregulation of signalling[102,103].

The recruitment of activated TLRs to membrane microdomains may also involve the binding of MAL that is pre-localized to regions of the plasma membrane that are enriched with PtdIns(4,5)P_2 (REF. 37). A recent study suggests that MAL may be promiscuous and may localize to other membrane systems that are enriched with different phosphoinositides. In the case of TLR9, MAL that is targeted by this mechanism to endosomes promotes the assembly of Myddosomes in response to natural ligands[104].

The coupling of activation and aggregation into microdomains may underlie the cooperative assembly of the post-receptor scaffold and the observed synergy of the TLR2 and TLR4 signalling processes[105]. Myddosome stoichiometry implies that activated TLR2 and TLR4 may co-assemble into a common scaffold, thereby integrating signals from simple and more complex bacterial lipids.

Conclusions and perspectives

Despite substantial recent progress, our understanding of the molecular mechanisms and dynamics of TLR signalling remains incomplete. Unresolved issues include the stoichiometry of signalling *in vivo*, the nature of the allosteric processes that are essential for regulation and the importance of lipid microdomains. Progress in these areas will be facilitated by the development of new methodologies, including super-resolution and single-molecule imaging[106–108], and an interdisciplinary approach involving low and high resolution structural techniques.

These further studies should facilitate the development of new therapies that are directed at the innate signalling network in autoimmune, inflammatory and infectious diseases. The potential of this approach is illustrated by existing small-molecule agonists of TLR7 that have shown efficacy in the treatment of viral infections, such as human papillomavirus[109]. In addition, a novel TLR8 agonist VTX-2337 has shown promise in Phase I clinical trials of metastatic head and neck cancers, and it has now entered Phase II clinical trials[110]. By contrast, small-molecule antagonists of TLR8 effectively inhibit TNF production in synovial explants and could replace TNF-targeted biological agents in the treatment of rheumatoid arthritis[111]. Targeting of the post-receptor pathway is also feasible. For example, cell-permeable peptides that inhibit the interaction of TRIF and TRAM in the TLR4 pathway are effective in a mouse model of sepsis and thus are candidate therapeutics for human endotoxic shock[49].

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Competing interests statement
The authors declare no competing interests.

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